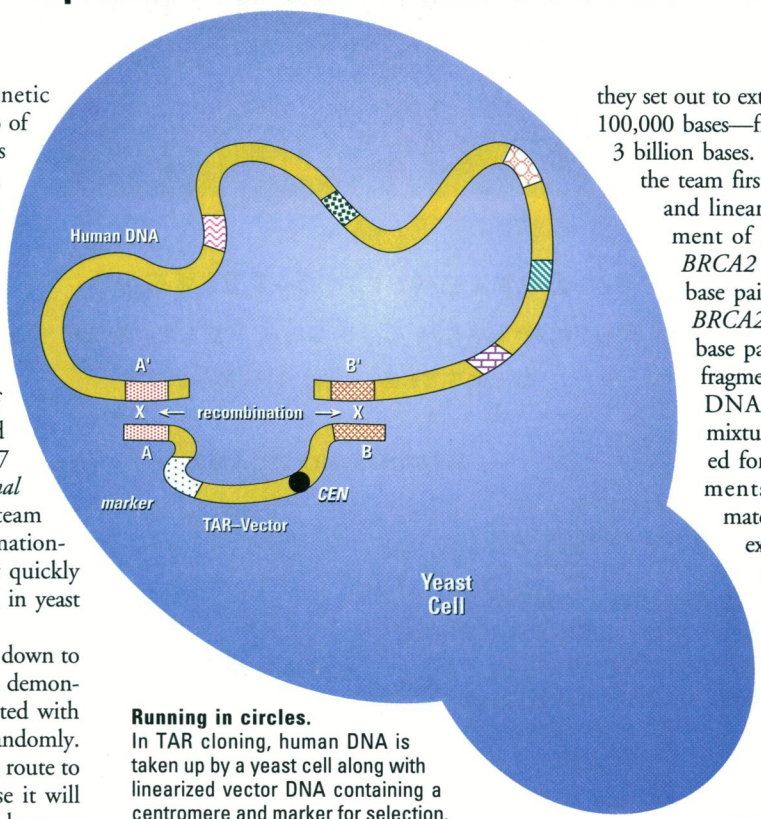
FAST-TRACK CLONING

In the lightning-paced world of genetic research, time is of the essence. A trio of NIEHS-based scientists have taken this adage to heart and recently reached an extraordinary milestone, cloning an entire single gene—the breast cancer gene *BRCA2*—in just two weeks' time. Michael Resnick, a scientist in the NIEHS Laboratory of Molecular Genetics, and Vladimir Larionov and Natasha Kouprina, visiting Russian scientists from the Institute of Cytology in St. Petersburg, published their new method in the 8 July 1997 issue of the *Proceedings of the National Academy of Sciences*. Last year, the team pioneered a method called transformation-associated recombination (TAR) for quickly cloning fragments of genetic material in yeast (see *EHP* 104:616–618).

In shaving a months-long process down to a mere two weeks, the team has also demonstrated that the clones can be generated with specificity and selectivity, instead of randomly. Their discovery is a significant step en route to mapping the human genome because it will enable other researchers to fill knowledge gaps in the genome faster and more accurately than has ever been possible. Equally important, however, is the cloning method's significance for human health: soon, this technique for rapid-fire recombinational recovery of human genes may open up new avenues toward gene therapy and gene discovery. The team's work has spawned a new gene isolation unit within the Laboratory of Molecular Genetics, which will work exclusively to isolate new genes in the future.

Isolating the Gene

Retrieving an exact gene copy so quickly is remarkable, considering that previous approaches took up to a year to complete. Until now, scientists who wanted to isolate and clone a gene or chromosomal fragment had to endure months of tedious analysis, sorting through a library of millions of fragments to find the exact ones they desired, then proceed through laborious and time-consuming mapping studies. "You had to go back and analyze every transformant—every clone—for whether you had what you wanted," says Resnick. To complicate the process even further, overlapping clones sometimes needed to be pieced together into a larger unit, or "contig," to achieve the desired clone. However, using the TAR cloning method in yeast, the NIEHS team decided to target specific genes. To do so, they set out to extract a single gene—roughly 100,000 bases—from the human genome of 3 billion bases. To isolate this single gene, the team first created a circular plasmid and linearized it to produce a fragment of DNA with this structure: *BRCA2* promoter sequence (669 base pairs)—centromere—HIS3—*BRCA2* last exon sequence (308 base pairs). They then added this fragment to total human genomic DNA, transformed the entire mixture into the yeast, and selected for HIS3$^+$ cells. Because segments on the human DNA matched the promoter and last exon "hooks" at each end of the plasmid, the segments together underwent intracellular recombination and produced the entire *BRCA2* gene, using information derived from only a tiny portion of the gene.



Running in circles. In TAR cloning, human DNA is taken up by a yeast cell along with linearized vector DNA containing a centromere and marker for selection. If segments of the human DNA correspond to hooks (A and B) on the plasmid, recombination will create a circular YAC. Source: Vladimir Larionov

At this point, the researchers obtained approximately 1,000 HIS3$^+$ clones and divided them into 33 pools, which they screened for *BRCA2* sequences by polymerase chain reactions. Their results confirmed that the entire gene was indeed present.

Larionov admits his initial reaction was disbelief when the first isolation was complete. "It was so exciting, but I refused to believe it for one month," he recalls. The team went on, though, to reproduce the results 12 times. "Only after that, I started to believe," Larionov says. And time after time, molecular analysis showed the quality of the clones to be very high. Larionov gives credit for their success in part to the team's collaboration with NIEHS scientific director Carl Barrett, whose work led to

the discovery of the *BRCA2* gene, as well as to Gregory Solomon, a researcher in the NIEHS's Laboratory of Molecular Carcinogenesis, which Barrett heads. "Solomon is an expert in molecular biotechnology; he helped us select what sequences we should target," says Larionov.

Sequences and Selectivity

Many scientists have contributed to the ongoing work of shaping up the human genome—work that should easily be completed within the next 5–10 years. In the past decade, researchers with the Human Genome Project have identified unique sequences called sequence tag sites approximately every 150 kilobytes along the genome, and have also sequenced short regions of nearly all the expressed genes producing expressed sequence tags. And even though most of the chromosomal DNA has been cloned, until recently no one had directly isolated genes that correspond to specific diseases, nor had anyone directly isolated specific chromosomal regions from genomic DNA.

"We're the only ones who have really explored the idea of achieving selectivity," Resnick says. "Prior to this, there were no really good, specific means for pulling out a gene. The best that had been done was to isolate chromosomes—to clone from the chromosomes. If you have a chromosome—that's 100 million bases—you still have to clone randomly," he says.

Though the team's work has been met with some surprise from other colleagues, the fundamental concept behind it is not new, Resnick says. He refers to that concept as the "TAR phenomenon"—that multiple molecules can be taken up by the yeast *Saccharomyces cerevisiae* during transformation, and that, if they have homology, or share identical sequences, then more than likely those molecules will undergo recombination.

The team also based its experiments on the knowledge that human DNA has sequences that can function as origins of replication in yeast so that when human DNA is combined with a plasmid lacking an origin, an artificial chromosome can be formed. The size of the resulting pieces of DNA did, however, come as a surprise to the team. They were able to retrieve pieces of genetic material that were 100,000–700,000 bases long.

Mapping the Missing Links

"The NIEHS team's discovery presents a tool scientists will eventually use to fill in the missing links in the human genome," says Eric Green, chief of the genome technology branch of the National Human Genome Research Institute. Green, whose own research has been dedicated to mapping and sequencing human chromosomes, likens the process of mapping the genome to the organization of a large book that contains the entire human genetic blueprint: "If you know a few words on page 48 and a few words on page 50, then here's a reliable method to find out what's on page 49," he says. "What they have done is isolate in a targeted fashion [one] page," he explains. Using this technique, scientists will be able to systematically fill in such "missing pages," and, in the near future, "finish the human blueprint very, very accurately," Green claims.

Larionov points out the time and cost savings advantages of using the TAR cloning method over random sequencing. With this new technique, he explains, "One person in one lab can accomplish this in two weeks, with no expensive equipment." This could replace the costly and time-consuming work of 20–30 people. It could, Larionov says, save millions of research dollars.

Researching the Gene–Disease Link

Besides filling gaps in the genome, the technique will also aid scientists in the search for disease-causing genes, according to Roger H. Reeves, associate professor in the department of physiology at Johns Hopkins University School of Medicine in Baltimore, Maryland, who has conducted genetic research on Down's syndrome and worked on the Human Genome Project. "It allows directed cloning of sequences from any individual," Reeves says. "Yeast artificial chromosome (YAC) library production is extremely expensive, time-consuming, and difficult," he explains. "It is not feasible to make additional YAC libraries from a variety of individuals, e.g., from each affected and unaffected member of a pedigree—[a chart of people within a family]—segregating a disease gene." According to Reeves, this technique would, however, "allow cloning of haplotypes [both the maternally and paternally inherited chromosomes belonging to an individual] containing mutant genes, greatly facilitating the search for genes responsible for human . . . disease."

Resnick agrees that TAR cloning opens the way to investigating genes and chromosomal regions directly from individuals. In such clinical studies, only 10–20 ml of a patient's blood would be necessary to isolate a particular gene. Now Resnick, Larionov, and Kouprina are investigating what they refer to as the "TAR cloning cycle," hoping to develop a technique that would be useful in transgenics. In this series of studies, they are working to isolate a gene, then return it to mammalian cells, a method that would be portable to other genetic diseases. In another study, they are seeking to confirm the concept of radial TAR cloning, meaning that a gene can be targeted even if only one sequence tag site is known.

Looking Ahead

The ability to isolate the *BRCA2* gene in its entirety will fuel further research into how this gene functions—about which little is known—as well as into the etiology of cancers associated with the gene. In future studies, the researchers hope to collaborate with Barrett's group to isolate and transfer the entire gene to tumor-derived human cell lines where it is nonfunctional, and thus facilitate a direct investigation into how the gene functions under the influence of its natural promoter. Eventually, the TAR cloning method could be used to rapidly clone mutant *BRCA2* forms from DNA extracted from clinical blood samples of breast cancer families, and then test those for function as well. So far, the team has isolated and cloned several other key disease genes as well, including the breast cancer gene *BRCA1*, *HPRT* (a gene related to a form of retardation known as Lesch-Nyhan syndrome), and rDNA (a group of genes involved in enzyme production).

Investigators in three other NIEHS laboratories are currently working to isolate more genes, says Larionov, while four NIH laboratories are targeting still more. That makes a total of seven more clones in the works, but Larionov won't be more specific, other than to say that the genes are human disease genes. With these sights in mind, Larionov has already lined up several postdoctoral researchers, including two from Japan, who will join him in the NIEHS's new gene isolation unit to pursue the next milestones on the path to gene discoveries.

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Suggested Reading

Larionov V, Kouprina N, Solomon G, Barrett JC, Resnick MA. Direct isolation of human *BRCA2* gene by transformation-associated recombination in yeast. Proc Nat Acad Sci USA 94:7384–7387 (1997).

Medlin J. A quicker way to clone. Environ Health Perspect 104:616–618 (1996).